

CLAIMS.

- 1) A DNA vector useful for stable transformation and expression of genes in plastids, where the gene to be expressed is inserted in the artificial intergenic region constituted by the combination of two 5' untranslated regions of plastid genes whose transcription naturally occurs in opposite directions and that belong to plants of different Classes or Divisions.
- 2) A DNA vector as claimed in claim 1, useful for achieving a high frequency of stable transformation and expression of genes in plastids of Angiosperm plants, with the following features:
 - a) contains an artificial intergenic region where it is possible to insert DNA sequences that encode for a polypeptide, under appropriated signals for its transcription and translation in plastids using transcription terminator sequences of non-plastid origin,
 - b) the artificial intergenic region is flanked by two genes whose transcription naturally occurs in opposite directions in plastid genomes, and whose nucleotide sequences are identical to the encoding sequences of *atpB* and *rbcL* genes belonging to plastids of Angiosperm plants from different Classes (dicotyledonous and monocotyledonous),
 - c) the artificial intergenic region is composed by: 5' regulatory sequences for transcription and translation of the *atpB* and *rbcL* genes; restriction sites for inserting genes of interest followed by a transcription terminator of non-plastid origin; and a second 5' regulatory region for the transcription and translation of an *rbcL* gene belonging to Angiosperm plastids from plants of a Class different to the Class of the previous *rbcL* gene.
- 3) A DNA vector as claimed in claim 2, comprising a border DNA sequence homologous to the sequence of plastid *rbcL* gene from tobacco or rice.
- 4) A DNA vector as claimed in claim 3, wherein said DNA sequence homologous to the *rbcL* gene comprises at least a fragment of the nucleotide sequence from position -291 to position +1233, starting from the translation initiation codon, of the tobacco *rbcL* gene.
- 5) A DNA vector as claimed in claim 4, wherein said border sequence homologous to the *rbcL* gene comprises the sequence SEQ.ID.NO: 4.
- 6) A DNA vector as claimed in claim 2, comprising a border DNA sequence homologous to the sequence of plastid *atpB* gene from rice or tobacco.
- 7) A DNA vector as claimed in claim 6, wherein said DNA sequence homologous to the *atpB* gene comprises at least a fragment of the nucleotide sequence from position -654 to position +1211, starting from the translation initiation codon, of the rice *atpB* gene.

- 8) A DNA vector as claimed in claim 7, wherein said border sequence homologous to the *atpB* gene comprises the sequence SEQ.ID.NO: 8.
- 9) A DNA vector as claimed in claim 2, wherein said transcription terminator of non-plastid origin present in the artificial intergenic region, is the bi-directional terminator *rmBT1T2* from *Escherichia coli* or an homologous thereof.
- 10) A DNA vector as claimed in claim 2, having inserted in the artificial intergenic region a gene that allows the selection of transplastomic plants.
- 11) A DNA vector as claimed in claim 10, wherein said selection gene can be eliminated by homologous recombination between direct repeated DNA sequences that border the gene.
- 12) A DNA vector as claimed in claim 11, wherein said selection gene encodes for a protein that allows plant cells to survive in presence of an antimicrobial compound.
- 13) A DNA vector as claimed in claim 12, wherein said antimicrobial compound belongs to the group of aminoglycoside antibiotics.
- 14) A DNA vector as claimed in claim 13, wherein said antibiotic compound is the Hygromycin B.
- 15) A DNA vector as claimed in claim 13, wherein said antibiotic compound is the Spectinomycin.
- 16) A DNA vector as claimed in claim 12, wherein said antimicrobial compound belongs to the group of sulfonamides.
- 17) A DNA vector as claimed in claim 16, wherein said antimicrobial compound is the Sulfadiazine.
- 18) A DNA vector as claimed in claim 10, wherein said selection gene encodes for a protein that allows plant cells to survive in presence of an herbicide compound.
- 19) A DNA vector as claimed in claim 18, wherein said herbicide compound belongs to the group of glutamine synthetase inhibitors.
- 20) A DNA vector as claimed in claim 19, wherein said herbicide compound is the Glufosinate (phosphinothricin).
- 21) A DNA vector as claimed in claim 19, wherein said herbicide compound is the Bialaphos.
- 22) A DNA vector as claimed in claim 18, wherein said herbicide compound belongs to the group of 5-enolpiruvil-shikimate-3-phosphate synthase (EPSPS) inhibitors.
- 23) A DNA vector as claimed in claim 22, wherein said herbicide compound is the Glyphosate (N-phosphomethyl glycine).

- 24) A DNA vector as claimed in claim 18, wherein said herbicide compound belongs to the group of inhibitors of folate synthesis.
- 25) A DNA vector as claimed in claim 24, wherein said herbicide compound is the Asulam (methyl- (4-aminobenzenesulphonyl)-carbamate).
- 5 26) A DNA vector as claimed in claim 18, wherein said herbicide compound belongs to the group of inhibitors of acetolactate synthase (ALS).
- 27) A DNA vector as claimed in claim 26, wherein said herbicide compound is a sulphonylurea.
- 28) A DNA vector as claimed in claim 26, wherein said herbicide compound is a
10 imidazolinone.
- 29) A DNA vector as claimed in claim 18, wherein said herbicide compound belongs to the group of inhibitors of photosystem II.
- 30) A DNA vector as claimed in claim 29, wherein said herbicide compound is the Bromoxynil (3,5-dibromo-4-hydroxybenzonitrile).
- 15 31) A DNA vector as claimed in claim 10, wherein said selection gene encodes for a protein that allows plant cells to survive in presence of a toxic compound.
- 32) A DNA vector as claimed in claim 31, wherein said toxic compound is the betaine aldehyde.
- 33) A DNA vector as claimed in claims 1 to 8, having the general structure described in
20 Figure1-A or variants thereof.
- 34) A DNA vector as claimed in claims 1 to 11, having the general structure described in Figure1-B or variants thereof.
- 35) A DNA vector as claimed in claims 1 to 11, having the general structure described in Figure1-C or variants thereof.
- 25 36) A DNA vector as claimed in claims 1 to 11, having the general structure described in Figure1-D or variants thereof.
- 37) A DNA vector as claimed in claims 1 to 11, having the general structure described in Figure1-E or variants thereof.
- 38) A DNA vector as claimed in claims 1 to 11, having the general structure described in
30 Figure1-F or variants thereof.
- 39) A DNA vector as claimed in claim 2, having in the second 5' untranslated region of *rbcL* gene a sequence functionally homologous to the binding site of the *Escherichia coli* lacI repressor.

- 40) A DNA vector having upstream of the translation initiation codon of *rbcL* gene a DNA sequence that allows to repress the transcription of this gene in non induction conditions, in order to provide the maintenance and multiplication of the vector in any host.
- 5 41) A DNA vector as claimed in claim 40, having in the 5' untranslated region of *rbcL* gene a sequence functionally homologous to the binding site of the *Escherichia coli* lacI repressor.
- 42) A DNA vector as claimed in claim 41, where the nucleotide sequence of said lacI binding site, corresponds at least in part to the sequence described in SEQ.ID.NO: 3.
- 10 43) A DNA vector as claimed in claims 1 to 11, having inserted in the artificial intergenic region one or more monocistronic or polycistronic transcriptional units under one or more plastid functional promoters.
- 44) A DNA vector as claimed in claims 12 to 17, having inserted in the artificial intergenic region one or more monocistronic or polycistronic transcriptional units under one or more plastid functional promoters.
- 15 45) A DNA vector as claimed in claims 18 to 38, having inserted in the artificial intergenic region one or more monocistronic or polycistronic transcriptional units under one or more plastid functional promoters.
- 46) A DNA vector as claimed in claims 43, 44 or 45, wherein at least one of the said plastid functional promoters is an *rbcL* promoter of the artificial intergenic region.
- 20 47) A DNA vector as claimed in claim 46, wherein the plastid functional promoters is the rice *rbcL* promoter comprised in sequence SEQ.ID.NO: 8.
- 48) A DNA vector as claimed in claims 43, 44 or 45, wherein at least one of the said plastid functional promoters is the *psbA** promoter comprised in the sequence SEQ.ID.NO: 16 or variants thereof.
- 25 49) A DNA vector as claimed in claim 48, having a mini-cistron functionally linked to said *psbA** promoter.
- 50) A DNA vector as claimed in claim 49, wherein said mini-cistron have a nucleotide sequence homologous to sequence described in SEQ.ID.NO: 17 or variants thereof.
- 51) A DNA vector for stable introduction and expression of genes in plastids that uses a mini-cistron for increasing the expression of genes introduced into transplastomic plants.
- 30 52) A DNA vector as claimed in claims 43, 44 or 45, wherein at least one gene of agricultural, veterinary, pharmaceutical, food or industrial interest is a component of the said transcriptional units.
- 53) A DNA vector as claimed in claim 52, wherein said gene of agricultural interest encodes for a protein with insecticide activity.

- 54) A DNA vector as claimed in claim 53, wherein said insecticide protein belongs to the family of Cry proteins from *Bacillus thuringiensis*.
- 55) A DNA vector as claimed in claim 53, wherein said insecticide protein is a protease inhibitor.
- 5 56) A DNA vector as claimed in claim 52, wherein said gene of agricultural interest encodes for a polypeptide with antimicrobial activity.
- 57) A DNA vector as claimed in claim 56, wherein said polypeptide belongs to one of the groups of the plant proteins related to pathogenicity (PR-proteins).
- 58) A DNA vector as claimed in claim 57, wherein said PR-protein is a glucanase.
- 10 59) A DNA vector as claimed in claim 57, wherein said PR-protein is a chitinase.
- 60) A DNA vector as claimed in claim 57, wherein said PR-protein is a thaumatin-like protein.
- 61) A DNA vector as claimed in claim 56, wherein said polypeptide is a ribosome inactivation protein (RIP).
- 15 62) A DNA vector as claimed in claim 52, wherein said gene of agricultural interest encodes for a protein that confers resistance to abiotic stress.
- 63) A DNA vector as claimed in claim 62, wherein said protein has choline oxidase activity.
- 64) A DNA vector as claimed in claim 52, wherein said gene of agricultural interest encodes for a protein that contributes to increase plant productivity.
- 20 65) A DNA vector as claimed in claim 64, wherein said protein is involved in the improvement of photosynthetic performance of the plant.
- 66) A DNA vector as claimed in claim 65, wherein said protein has fructose-1, 6-bisphosphate phosphatase (FBPase) activity.
- 67) A DNA vector as claimed in claim 65, wherein said protein has protoporphyrinogen
25 oxidase (PROTOX) activity.
- 68) A DNA vector as claimed in claim 65, wherein said protein has ribulose-1-5-bisphosphate carboxylase/oxygenase (RUBISCO) activity.
- 69) A DNA vector as claimed in claim 65, wherein said protein takes part directly or indirectly in the carbon fixation process of the plants.
- 30 70) A DNA vector as claimed in claim 52, wherein said gene of agricultural interest encodes for a protein that contributes to increase the post-harvest conservation of the plant products.
- 71) A DNA vector as claimed in claim 52, wherein said gene of food interest encodes for a protein that contributes to improve the nutritional quality of the plant products.

72) A DNA vector as claimed in claim 52, wherein said gene of veterinary or pharmaceutical interest encodes for a cytokine.

73) A DNA vector as claimed in claim 72, where said cytokine belongs to the family of interferons.

5 74) A DNA vector as claimed in claim 72, where said cytokine belongs to the family of interleukines.

75) A DNA vector as claimed in claim 52, wherein said gene of veterinary or pharmaceutical interest encodes for a polypeptide with regulatory activity on the immune response.

10 76) A DNA vector as claimed in claim 52, wherein said gene of veterinary or pharmaceutical interest encodes for a polypeptide with hormonal activity.

77) A DNA vector as claimed in claim 76, wherein said polypeptide with hormonal activity is the insulin.

78) A DNA vector as claimed in claim 76, wherein said polypeptide with hormonal activity is a growth hormone.

15 79) A DNA vector as claimed in claim 76, wherein said polypeptide with hormonal activity is a somatotropic hormone.

80) A DNA vector as claimed in claim 76, wherein said polypeptide with hormonal activity is a gonadotropic hormone.

20 81) A DNA vector as claimed in claim 52, wherein said gene of veterinary or pharmaceutical interest encodes for a cell proliferation factor.

82) A DNA vector as claimed in claim 81, wherein said cell proliferation factor is the epidermic growth factor (EGF).

83) A DNA vector as claimed in claim 52, wherein said gene of veterinary or pharmaceutical interest encodes for a polypeptide with hematopoietic activity.

25 84) A DNA vector as claimed in claim 52, wherein said gene of veterinary or pharmaceutical interest encodes for a cell receptor.

85) A DNA vector as claimed in claim 52, wherein said gene of veterinary or pharmaceutical interest encodes for a protease inhibitor.

30 86) A DNA vector as claimed in claim 52, wherein said gene of veterinary or pharmaceutical interest encodes for a polypeptide with trombolytic activity.

87) A DNA vector as claimed in claim 86, wherein said polypeptide is the streptokinase.

88) A DNA vector as claimed in claim 86, wherein said polypeptide is the tissular plasminogen activator (t-PA).

89) A DNA vector as claimed in claim 52, wherein said gene of veterinary or pharmaceutical interest encodes for a vaccine antigen.

90) A DNA vector as claimed in claim 89, wherein said vaccine antigen belongs to a virus.

5 91) A DNA vector as claimed in claim 90, wherein said vaccine antigen belongs to hepatitis virus.

92) A DNA vector as claimed in claim 91, wherein said vaccine antigen is the hepatitis B surface antigen.

93) A DNA vector as claimed in claim 91, wherein said vaccine antigen belongs to the hepatitis A virus.

10 94) A DNA vector as claimed in claim 91, wherein said vaccine antigen belongs to the hepatitis C virus.

95) A DNA vector as claimed in claim 90, wherein said vaccine antigen belongs to the foot and mouth disease virus (FMDV).

15 96) A DNA vector as claimed in claim 90, wherein said vaccine antigen belongs to the human immunodeficiency virus (HIV).

97) A DNA vector as claimed in claim 89, wherein said vaccine antigen belongs to a bacterium.

98) A DNA vector as claimed in claim 89, wherein said vaccine antigen belongs to a protozoan.

20 99) A DNA vector as claimed in claim 52, wherein said gene encodes for a fragment of the variable region of an immunoglobulin.

100) A DNA vector as claimed in claim 52, wherein said gene encodes for a multimeric protein.

25 101) A DNA vector as claimed in claim 100, wherein said multimeric protein is an immunoglobulin.

102) A DNA vector as claimed in claim 100, wherein said multimeric protein is a hormone.

103) A DNA vector as claimed in claim 100, wherein said multimeric protein is a vaccine antigen.

104) A DNA vector as claimed in claim 100, wherein said multimeric protein is an enzyme.

30 105) A DNA vector as claimed in claim 100, wherein said multimeric protein is a cell receptor.

106) A DNA vector as claimed in claim 52, wherein said gene of industrial interest encodes for a protein component of a biopolymer.

- 107) A DNA vector as claimed in claim 52, wherein said gene of industrial interest encodes for an enzyme.
- 108) A DNA vector as claimed in claim 107, wherein said enzyme is a protease.
- 109) A DNA vector as claimed in claim 107, wherein said enzyme is a lipase.
- 5 110) A DNA vector as claimed in claim 107, wherein said enzyme is an isomerase.
- 111) A DNA vector as claimed in claim 107, wherein said enzyme has glycosil-hydrolase activity.
- 112) A DNA vector as claimed in claim 111, wherein said enzyme with glycosil-hydrolase activity is a levansucrase.
- 10 113) A DNA vector as claimed in claim 111, wherein said enzyme with glycosil-hydrolase activity is an invertase.
- 114) A DNA vector as claimed in claim 111, wherein said enzyme with glycosil-hydrolase activity is a levanase.
- 115) A DNA vector as claimed in claim 111, wherein said enzyme with glycosil-hydrolase activity is a dextranase.
- 15 116) The culture of vegetable cells in presence of cytokinins, before introducing into them any of the DNA vectors claimed in claims 1 to 115, to increase the frequency of production of transplastomic plants.
- 117) The use of kinetin as claimed in claim 116.
- 20 118) The Angiosperm transplastomic plants stably transformed with any of the DNA vectors claimed in claims 1 to 115.
- 119) The progeny of transplastomic plants of claim 118.
- 120) The transplastomic plants of claims 118 and 119 that stably express at least one of the genes present in the DNA vector used for transformation.
- 25 121) The transplastomic plants of claims 118 and 119 with hybrid atpB and/or rbcL proteins.
- 122) The culture of transplastomic plants of claim 120.
- 123) The culture of cells of the transplastomic plants of claim 120.
- 124) The purification and use of the protein, or proteins, that produce the cells of the transplastomic plants of claim 120 as a result of the expression of said gene or genes.
- 30 125) The transplastomic plants of claims 118 to 121 that are Angiosperms.
- 126) The transplastomic plants of claim 125 that are dicotyledonous.
- 127) The transplastomic plants of claim 126 that are solanaceous.
- 128) The transplastomic plants of claim 127 that belong to one of the following species: tobacco, tomato or potato.

129) The transplastomic plants of claim 125 that are monocotyledonous.

130) The transplastomic plants of claim 129 that are gramineous.

131) The transplastomic plants of claim 130 that belong to one of the following species: rice, sugar cane, maize, wheat or barley.